JOURNAL OF
THE LEPIDOPTERISTS' SOCIETY

Volume 56 2002 Number 4

Journal of the Lepidopterists' Society
56(4), 2002, 193–198

EXPERIENCE-RELATED CHANGES IN THE BRAIN OF *AGRAULIS VANILLAE* (L.) (NYMPHALIDAE)

VADIM KROUTOV
123 Bartram Hall, Department of Zoology, University of Florida, Gainesville, Florida 32611, USA

ROGER L. REEP
College of Veterinary Medicine, Health Science Center, P.O. Box 100144 Gainesville, Florida 32610-0144, USA

AND

TOK FUKUDA
Center for Medical, Agricultural and Veterinary Entomology, Agricultural Research Service,
U.S. Department of Agriculture, Gainesville, Florida 32604, USA

ABSTRACT. In the brain of *Agraulis vanillae*, the size of the brain regions involved in the processing of olfactory information was found to depend on the butterfly's experience. Butterflies collected in nature have olfactory glomeruli and mushroom body calyxes of larger relative size than do butterflies reared and kept in the laboratory in isolation from normal environmental stimuli. No size difference was found in the optic lobes or the central body in either males or females.

Additional key words: mushroom body, neuropil, olfactory lobes.

The brain of an insect is the principal associative center of the body. It receives sensory information from a variety of sense organs, processes it and controls all functions of the organism, including complex forms of behavior. Several regions of the brain differing in morphology and function are recognized and referred to as neuropils (Fig. 1). Neuropils are the centers of the regions and are formed by a complex of densely packed nerve fibers. The neurons, which compose a region, lie at its periphery. On histological sections of the brain neuropils appear as much denser, darker than the rest of the brain areas.

The neuropils of particular significance in the processing of information in insect brains are the mushroom bodies and antennal lobes. Mushroom bodies receive signals from different sense organs and experiments on *Drosophila* (Heisenberg et al. 1985, Han et al. 1992) and *Apis* (Erber et al. 1980, Menzel et al. 1974, Hammer & Menzel 1998) suggest that they are implicated in olfactory memory formation. They are composed of three types of cells: cells that direct signals to the mushroom bodies, cells that deliver signals from the mushroom bodies to other parts of the nervous system, and the intrinsic cells (Kenyon cells) that connect the first two types between themselves. The Kenyon cells occupy the area around the mushroom body neuropil.

All information from the organs of smell (olfactory organs) is received in another brain region: antennal lobes, which are critically important in the delivery of olfactory information to the mushroom bodies. Antennal lobes are composed of a series of neuropils-olfactory glomeruli, which receive and process olfactory signals from the antennae.

Insect species with complex and flexible behavior possess well-developed mushroom bodies and antennal lobes, and larger insects have larger brains and more complex histological brain structure and generally exhibit greater complexity of behavior (Goossen 1949, Bernstein & Bernstein 1969). The largest mushroom bodies (relative to the rest of the brain) are found in social Hymenoptera. The morphological plas-

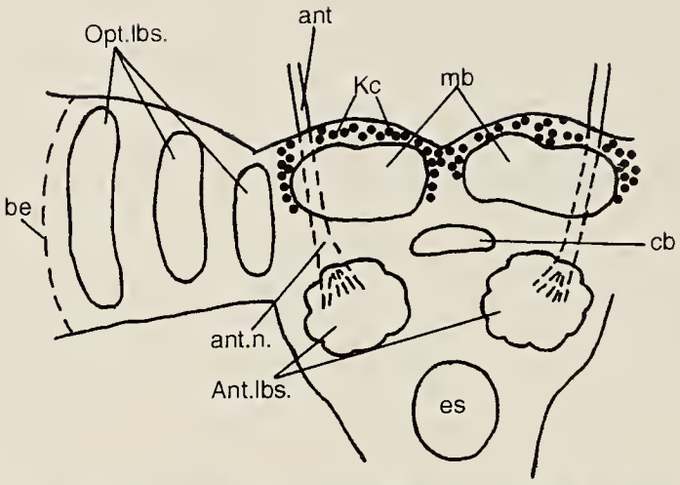

FIG. 1. Diagram of the butterfly brain showing the most important neuropils. **Opt.lbs.**—optic lobes, **Ant.lbs.**—antennal lobes, **mb**—mushroom bodies, **Kc**—Kenyon cells, **cb**—central body, **be**—back of the eye, **ant.n.**—antennal nerve, **ant.**—antenna, **es**—esophagus.

ticity of these brain structures has been demonstrated in bees (Withers et al. 1993, Winnington et al. 1996, Robinson 1998) and ants (Gronenberg et al. 1996). Mushroom bodies increase in size when these insects begin to perform complex and behaviorally more demanding tasks. Neuropil growth related to behavioral changes has also been observed in non-social insects, such as fruit flies and rove beetles (Bieber & Fuldner 1979, Technau 1984, Heisenberg et al. 1995). This growth was found to represent the further arborization and proliferation of existing brain cells, and not the production of new neurons.

Flexibility of behavior and learning have been demonstrated in different species of Lepidoptera (Swihart & Swihart 1970, Papaj 1986, Weiss 1995, 1997, Hartlieb 1996, Fan et al. 1997). Butterflies and moths have well-developed mushroom bodies (Ali 1974, Sivinsky 1989), and large antennal lobes (Matsumoto & Hildebrand 1981). Both olfactory and visual learning have been described in *Agraulis vanillae* (Weiss 1995, Kroutov et al. 1999).

Here we studied brain morphology in two groups of *Agraulis*. One group comprised butterflies collected in nature ("experienced" group) and the other group was reared and maintained in the laboratory in isolation from normal environmental stimuli ("naïve" group). We investigated the hypotheses that the sizes of brain structures involved in information processing and learning vary according to the individual experience of butterflies, and that such structures should be larger in butterflies exposed to various environmental stimuli than in butterflies deprived of those.

MATERIALS AND METHODS

Adults and larvae of *Agraulis vanillae* were collected in Gainesville, Florida. All butterflies used in experiments were collected during the 3–4 day period of the abundance peak of the species. Larvae were reared in the laboratory on their natural host-plant *Passiflora incarnata* (L.), picked in the same area where the larvae were found. Laboratory reared adults spent 48 hours after eclosion in 25 × 25 × 25 cm screen cages. The laboratory conditions were 25°C, 65% relative humidity, L:D 16:8 h. Butterflies were fed a 25% sugar solution.

For the preparation of the histological specimens butterfly heads were removed and fixed in Bouin's fixative, prepared 24 hours prior to usage, for 2 days. They were then rinsed in 70% ethanol and embedded in paraffin. Heads of 16 reared males, 10 reared females, 17 wild males and 22 wild females were sectioned. The frontal microtome sections were 10 µm thick and were stained with hematoxylin-eosin.

Volumetric analysis was performed with an AIS/C image analysis system (Imaging Research, Inc.) interfaced to a Zeiss Axiophot microscope via a Dage 72 CCD camera. The following areas were measured in selected spaced sections on both sides of the brain: whole brain, antennal lobes, olfactory glomeruli, optic lobes, central body, mushroom body calyces, and the regions occupied by Kenyon cells. When areas were measured, this was done without awareness of the group to which that individual belonged. The volume of a brain structure was calculated using the formula

$$\mathrm{Vol}_{(\mathrm{object})} = \sum_{i=1}^{n} A_i \times t \times N$$

where **n** is the number of sections on which measurements were made, **A** is the area of a measured section, **t** is the distance between adjacent sections (e.g., section thickness), and **N** is the number of sections represented by the section $\mathbf{A_i}$. Between 10 and 20 evenly spaced sections were used to determine the volume of each region. This corresponded to 50–100% of all the sections containing each measured structure. The relative volume of each brain structure was calculated as a percentage of the volume of the whole brain.

For statistical analysis of the data a fixed effects linear model (ANOVA) was fit with PROC GLM (SAS v.8). That is, size was modeled as a function of the fixed effects 'brain region', 'butterfly gender' and 'butterfly group' ("experienced", "naïve" and "control"). All relevant assumptions such as constant variance and normality were formally assessed. Due to the large number of multiple Bonferroni comparisons we tested at the 0.01 level of significance throughout.

To exclude the possible effect of age on the changes in *Agraulis* brain, a control group of 10 males and 10 females, reared in the laboratory was kept in cages for 20–25 days after eclosion under the same conditions as described for the experimental group. The heads of

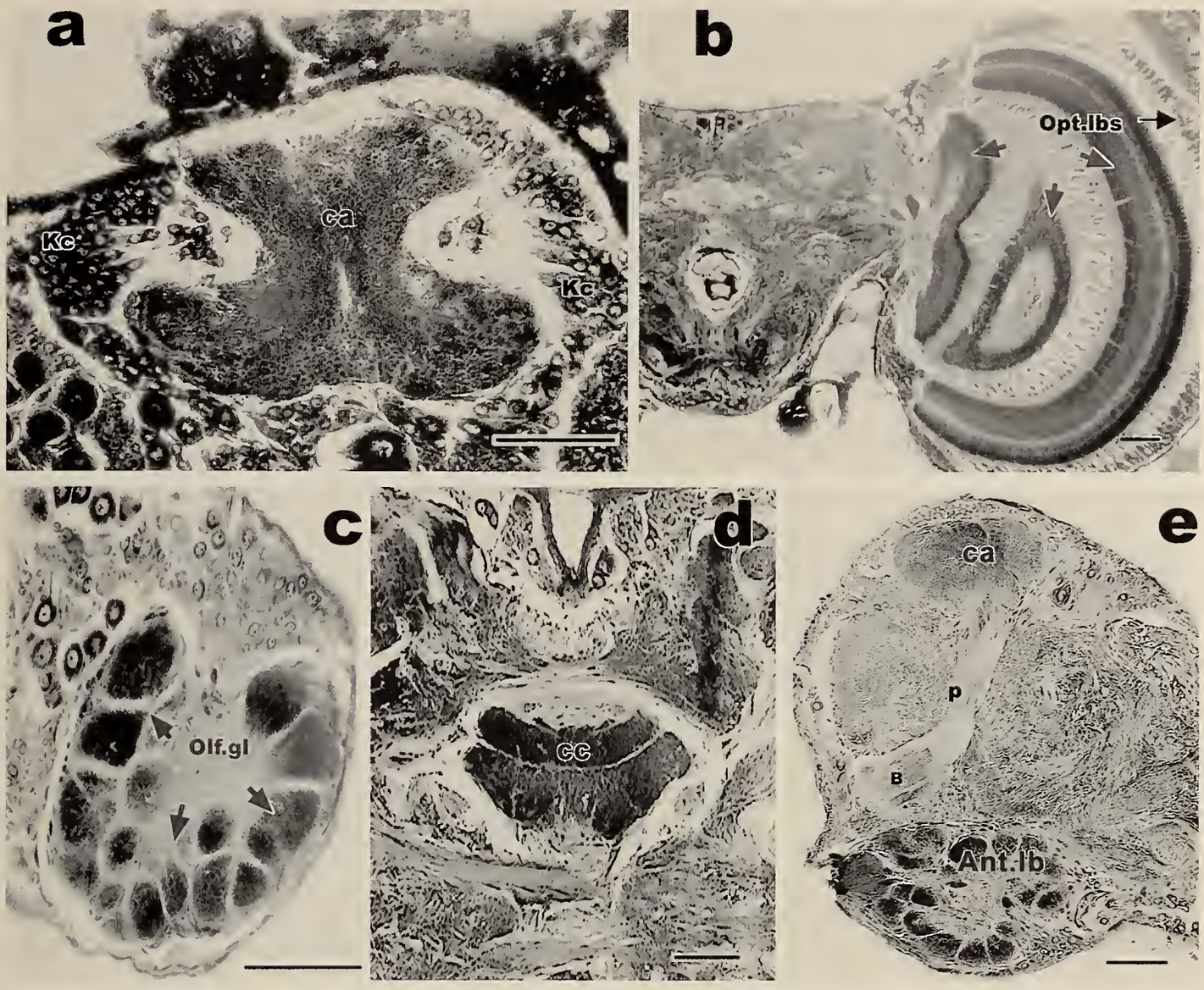

FIG. 2. Sections of the brain of *Agraulis vanillae*. **a:** mushroom body calyx (ca) and Kenyon cells (Kc); **b:** optic lobes (Opt.lbs); **c:** antennal lobe with glomeruli (Olf.gl.); **d:** central body (cc); **e:** mushroom body—calyx (ca), pedunculus (p), β-lobe (B) and antennal lobe (Ant.lb.). **a,b,c,d**—frontal sections, **e**—sagittal section. Scale bars—100 μm.

control butterflies were sectioned, sections stained and brains measured as described above.

RESULTS

Figure 2 shows the sections of the measured brain structures in *Agraulis vanillae*. Most of the regions exhibit clearly defined boundaries. Because of the absence of a clear boundary between the mushroom body's pedunculus and lobes, and the surrounding neuropil, attributable to the staining method chosen for the study, only mushroom body calyces were measured.

Whole brain volume of *Agraulis* showed no significant variation according to group (Fig. 3). There was found to be a significant interaction of gender *group* brain region ($p < 0.0001$). Multiple pairwise comparisons revealed the following patterns: "experienced" individuals of both sexes exhibited significantly larger mushroom bodies and olfactory glomeruli than did "naïve" or "control" individuals (Fig. 4; Table 1). The relative volume of mushroom body calyces in "experienced" butterflies was greater than in "naïve" ones by 36% in males, and by 38% in females. Olfactory glomeruli were larger in "experienced" *Agraulis* by 48% in males, and 24% in females.

The Kenyon cells region and antennal lobes showed mixed outcomes. Within the Kenyon cells region, there were no significant differences in volume among the male groups, but "experienced" females exhibited smaller volumes than did "controls". For the antennal lobes, "experienced" males have larger volumes than do "naïve" males. There were no differences among the female groups. The central body and optic lobe regions exhibited no significant difference for any pairwise comparison.

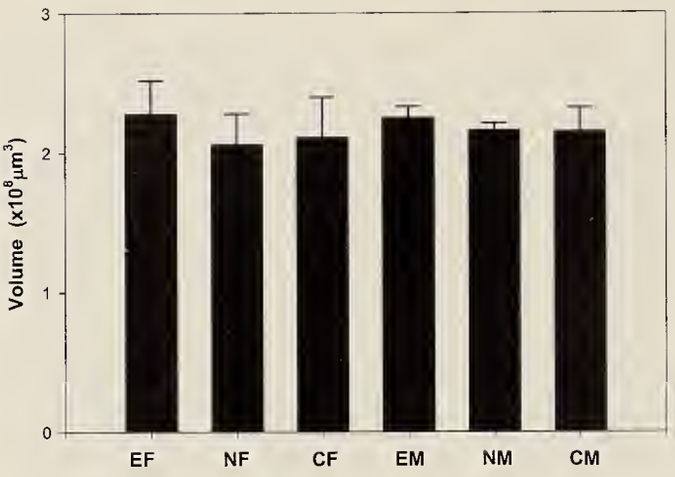

FIG. 3. Whole brain volume in *Agraulis vanillae*. **EF**—"experienced" females, **NF**—"naïve" females, **CF**—"control" females, **EM**—"experienced" males, **NM**—"naïve" males, **CM**—"control" males.

For most brain regions assessed, there was no significant difference between male and female volumes. However, the antennal lobes exhibited the following pattern: "naïve" and "control" females have larger antennal lobes than their male counterparts ($p < 0.0001$ in each case), but "experienced" females do not differ significantly from "experienced" males.

For all brain regions, the "naïve" and "control" groups exhibited no significant differences in volume, within males or females.

DISCUSSION

The results of our research demonstrate that in *Agraulis* differences in experience are correlated with changes in the volume of several of its brain regions involved in sensory information processing and memory formation. During their adult stage (2–4 weeks), *Agraulis vanillae* butterflies must perform various activities, the success of which can be enhanced by learning. Location of feeding sites with flowers that offer sufficient nectar reward, and recognition of potential danger are of importance to both sexes. Female *Agraulis* need to find suitable host-plants on which to lay eggs. This involves not only recognition of the proper plant amongst a variety of other plants, but also memory of the location of the host *Passiflora* patch, because butterflies of this species utilize vast habitats and linger at one spot for no longer than is necessary to complete either feeding or egg-laying. Males, in turn, need to locate the host-plant area to encounter females and mate.

Detailed analysis of the captivity conditions and their specific influence on *Agraulis*'s experience, learning and associated morphological changes in its brain was not attempted. However, it seems evident that captive laboratory-reared butterflies would have a greatly reduced range of external stimuli, being deprived of space, visual stimuli, contacts with host-plant, flowers and sex partners. It was also impossible to determine precisely the age of "experienced" butterflies, collected in nature. But because we collected them in a period of 10–14 days after the beginning of their abundance peak, we can estimate all collected butterflies to be of approximately the same age.

Generally, measured brain structures were larger (relative to the volume of the whole brain) in "experienced" butterflies. But no difference in the relative volume of optic lobes and central body was recorded between two groups of *Agraulis*. The most dramatic increases in relative volume occurred in the mushroom bodies and olfactory glomeruli, whereas no size difference in optic lobes between "experienced" and "naïve" butterflies was observed. Therefore, olfactory stimuli may be of primary importance in driving the structural changes in the *Agraulis* brain. Although butterflies reputedly rely heavily on visual stimuli (Swihart 1970, Silberglied 1979, 1984), and *Agraulis* is capable of visual as well as olfactory learning (Weiss 1995), their optic lobes only pass visual information to the central brain, where processing and integration of this information takes place. Therefore such a result is predictable.

The relative decrease in volume of the Kenyon cells region is rather hard to explain. However, because there was no change in the whole brain volume, this region's relative decrease could represent an actual compression of the Kenyon cell clusters by the ex-

TABLE 1. Relative volumes of brain regions as percentage of the whole brain volume in *Agraulis vanillae*. Within each box, different small case letters indicate significant differences, whereas the same letters indicate no difference.

	Mushroom body calyx	Olfactory glomeruli	Kenyon cells region	Antennal lobes	Central body	Optic Lobes
"Experienced" males	2.01 ± 0.08 a	1.45 ± 0.09 a	0.69 ± 0.07 a	3.74 ± 0.35 b	0.61 ± 0.09 a	64.4 ± 3.0 a
"Naïve" males	1.48 ± 0.05 b	0.98 ± 0.05 b	0.75 ± 0.05 a	3.41 ± 0.10 a	0.68 ± 0.04 a	69.7 ± 8.3 a
"Control" males	1.38 ± 0.07 b	0.98 ± 0.09 b	0.72 ± 0.04 a	3.50 ± 0.10 ab	0.66 ± 0.05 a	65.1 ± 2.3 a
"Experienced" females	2.18 ± 0.10 a	1.44 ± 0.08 a	0.56 ± 0.03 a	3.90 ± 0.10 a	0.63 ± 0.07 a	61.8 ± 2.6 a
"Naïve" females	1.58 ± 0.05 b	1.16 ± 0.05 b	0.73 ± 0.05 ab	4.00 ± 0.20 a	0.64 ± 0.03 a	62.4 ± 2.2 a
"Control" females	1.58 ± 0.12 b	1.11 ± 0.04 b	0.84 ± 0.08 b	4.00 ± 0.12 a	0.66 ± 0.02 a	61.1 ± 1.9 a

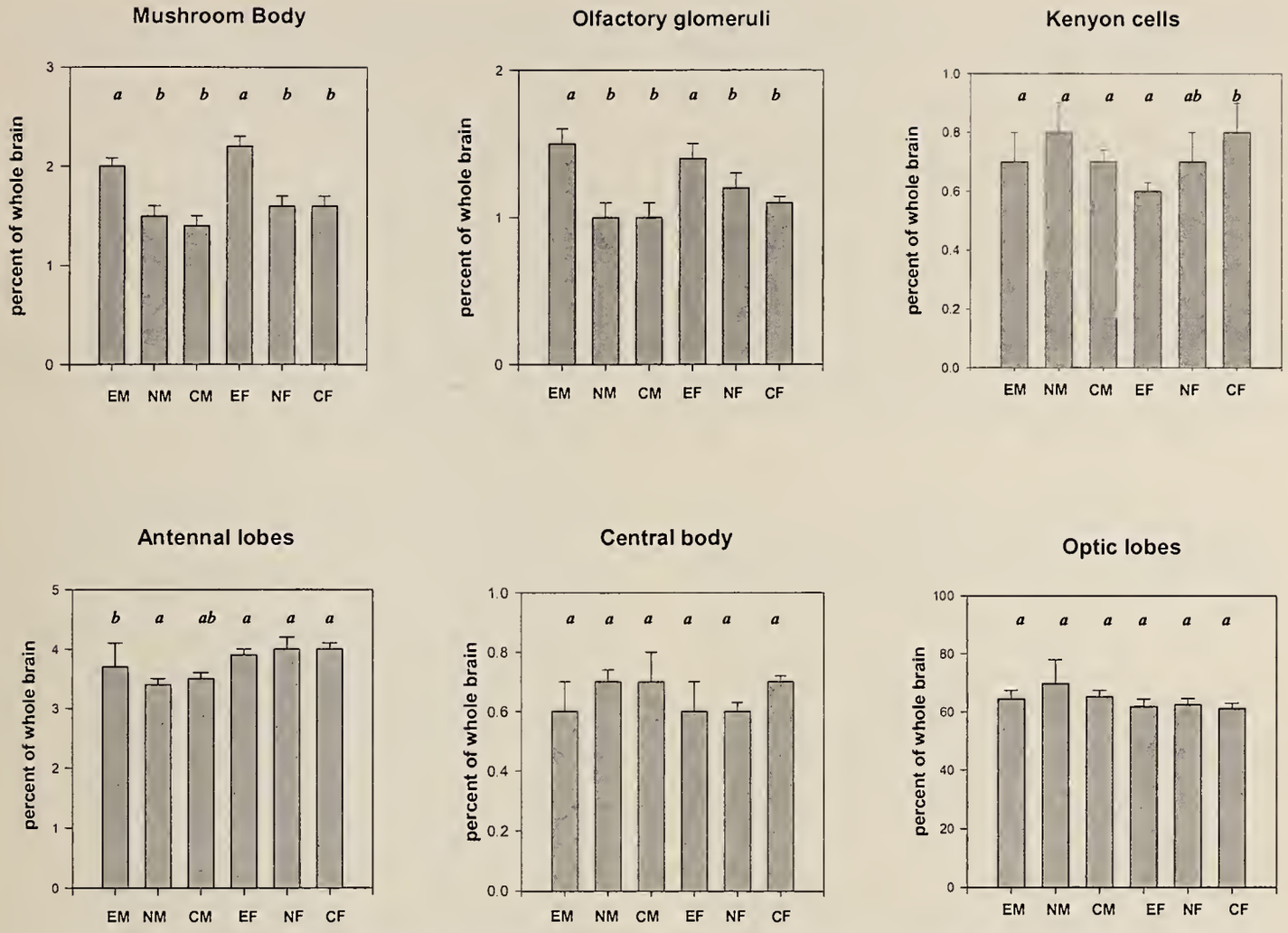

FIG. 4. Relative volumes of brain regions as percentage of the whole brain volume in *Agraulis vanillae*. **EM**—"experienced" males, **NM**—"naïve" males, **CM**—"control" males. **EF**—"experienced" females, **NF**—"naïve" females, **CF**—"control" females. Different small case letters indicate significant differences, whereas the same letters indicate no difference for each sex.

panding mushroom body calyces. This problem could be addressed by more detailed experimental analysis, for example assessment of cell packing density.

The data presented here are similar in many ways to those of studies in other species of insects, which measured the size differences in brain regions caused by different experience and behavioral repertoire. As in the present study, an increase in relative volume of mushroom bodies, and decrease in relative volume of the Kenyon cells region were reported for ants (Gronenberg et al. 1996) and bees (Withers et al. 1993). Also, there was no increase in the relative volume of the optic lobes in either of these insects. Olfactory glomerular volume was found to differ between 1-day-old and nurse bees (larger in nurses), but the increase was not maintained in foragers. For rove beetles mushroom body volume increase and no changes in optic lobe volume were recorded (Bieber & Fuldner 1979).

The sexual dimorphism found in the reorganization of some brain structures in *Agraulis*, namely the difference in olfactory glomeruli volume between "naïve" and "experienced" males being twice as great as that in females, can perhaps be explained by the differences in behavior of males and females. Females need to locate host-plants and determine their suitability for oviposition, and males need to search for females and recognize proper chemical cues from suitable partners. Thus, each sex may rely on different environmental stimuli. The change in the intensity of these stimuli may effect butterflies of different sexes differently, and cause the observed dissimilarity in the brain reconstruction. This dimorphism corresponds with our earlier findings in *Agraulis* learning (Kroutov et al. 1999), where different learning capability was recorded for the two sexes.

Measurements in the control group show that morphological changes in the brain of *Agraulis vanillae* are not age-related, but experience-related, since the relative volumes of the studied brain structures in 2-day ("naïve") and 20–25-day ("control") butterflies were not significantly different. These changes occur in only

a few brain compartments that are noted for their role in information processing and learning in insects. This further supports the hypothesis that growth of these brain regions is related to learning experience and behavioral complexity of a butterfly.

Further experiments involving the manipulation of various elements of the environment may lead to a better understanding of the exact relation between particular types of information, how they are processed, and changes they cause in the brain of *Agraulis vanillae*. It would be especially interesting to analyze the specific effects of various environmental "deprivations" and, inverted, the effect of additional stimuli on the changes in *Agraulis'* brain structures.

ACKNOWLEDGMENTS

We thank Dr. M. S. Mayer for his helpful comments on our paper, Scott Whittaker for his help with the preparation of the illustrations and Galin Jones for helping us with the statistical analysis of our data.

LITERATURE CITED

ALI, F. A. 1974. Structure and metamorphosis of the brain and suboesophageal ganglion of *Pieris brassicae* (L.) (Lepidoptera: Pieridae). Trans. R. Ent. Soc. Lond. 125 (4):363–412.

BERNSTEIN, S. & R. BERNSTEIN. 1969. Relationships between foraging efficiency and the size of the head and component brain and sensory structures in the red wood ant. Brain Research 16:85–104.

BIEBER, M. & D. FULDNER. 1979. Brain growth during the adult stage of a holometabolous insect. Naturwissenschaffen 66:426.

ERBER, J., T. MASUHR & R. MENZEL. 1980. Localization of short-term memory in the brain of the bee, *Apis mellifera*. Physiol. Entomol. 5:343–358.

FAN, R.-J., P. ANDERSON & B. S. HANSSON. 1997. Behavioural analysis of olfactory conditioning in the moth *Spodoptera littoralis* (Bsdv.) (Lepidoptera: Noctuidae). J. Exp. Biol. 200:2969–2976.

GOOSSEN, H. 1949. Untersuchungen an Gehirnen verschieden grosser, jeweils verwandter Coleopteren- und Hymenopteren-Arten. Zool. Jb. Abt. Allg. Zool. 62:1–64.

GRONENBERG, W., S. HEEREN & B. HOLLDOBLER. 1996. Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. J. Exp. Biol. 199:2011–2019.

HAMMER, M. & R. MENZEL. 1998. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learning and Memory 5:146–156.

HAN, P. L., L. R. LEVIN, R. R. REED & R. L. DAVIS. 1992. Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. Neuron 9:619–627.

HARTLIEB, E. 1996. Olfactory conditioning in the moth *Heliothis virescens*. Naturwissenschaffen 83:87–88.

HEISENBERG, M., A. BORST, S. WAGNER & D. BYERS. 1985. *Drosophila* mushroom body mutants are deficient in olfactory learning. J. Nerogenet. 2:1–30.

HEISENBERG, M., M. HEUSIPP & CH. WANKE. 1995. Structural plasticity in the *Drosophila* brain. J. Neurosci. 15 (3):1951–1960.

KROUTOV, V., M. S. MAYER & T. C. EMMEL. 1999. Olfactory conditioning of the butterfly *Agraulis vanillae* (L.) (Lepidoptera, Nymphalidae) to floral but not host-plant odors. J. Insect Behav. 12 (6):833–843.

MATSUMOTO, S. G. & J. G. HILDEBRAND. 1981. Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. Proc. R. Soc. Lond. B 213:249–277.

MENZEL, R., J. ERBER & T. MASUHR. 1974. Learning and memory in the honey bee, pp. 195–218. *In* Browne, L. B. (ed.), Experimental analysis of insect behavior. Springer, Berlin.

PAPAJ, D. R. 1986. Conditioning of leaf-shape discrimination by chemical cues in the butterfly, *Battus philenor*. Anim. Behav. 34:1281–1288.

ROBINSON, G. E. 1998. From society to genes with the honey bee. Amer. Scientist 86 (5):456–462.

SILBERGLIED, R. E. 1979. Communication in the ultraviolet. A. Rev. Ecol. Syst. 10:373–398.

———. 1984. Visual communication and sexual selection among butterflies, pp. 207–223. *In* Vane-Wright, R. I. & P. K. Ackery (eds.), The biology of butterflies. Academic Press.

SIVINSKI, J. 1989. Mushroom body development in nymphalid butterflies: a correlate of learning? J. Insect Behav. 2 (2):277–283.

SWIHART, C. A. & S. L. SWIHART. 1970. Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius*. Anim. Behav. 19:156–164.

SWIHART, S. L. 1970. The neural basis of colour vision in the butterfly, *Papilio troilus*. J. Insect Physiol. 16:1623–1636.

TECHNAU, G. 1984. Fiber number in the mushroom bodies of adult *Drosophila melanogaster* depends on age, sex and experience. J. Neurogenet. 1:113–126.

WEISS, M. R. 1995. Associative colour learning in a nymphalid butterfly. Ecol. Entomol. 20:298–301.

———. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. Anim. Behav. 53:1043–1052.

WINNINGTON, A. P., R. M. NAPPER & A. R. MERCER. 1996. Structural plasticity of identified glomeruli in the atennal lobes of the adult worker honey bee. J. Comp. Neurol. A 365:479–490.

WITHERS, G., S. FAHRBACH & G. E. ROBINSON. 1993. Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238–240.

Received for publication 6 March 2001; revised and accepted 21 February 2002.